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DESCRIPTION

ADENOVIRUS VECTOR

Technical Field

The present invention relates to an adenovirus vector that is utilized when, for example, transfecting a gene of interest into a target cell.

Background Art

In 1953, adenoviruses were separated from a culture solution of pediatric tonsil or adenoid cells. The existence of 80 or more adenovirus serotypes that are infected with humans, birds, cattle, monkeys, dogs, mice, or pigs as a host has been discovered to date. Up to the present, 51 or more types of adenovirus serotypes that are infected with humans as a host have been discovered, and adenovirus type 2 and type 5 are used as vectors for gene therapy.

Adenovirus type 5 is a non-enveloped and regular icosahedron with 252 capsomeres. Among them, the 12 capsomeres located at the peak of the icosahedron are referred to as "pentons" (composed of Penton bases and fibers) having projected structures and the other 240 capsomeres are referred to as "hexons." Viruses infiltrate (infect) cells as follows. Fibers bind to the CAR receptors (for details, please refer to Bergelson J M et al., Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5, Science 275: 1320-1323, 1997) and the RGD motifs of the penton bases then bind to the integrins on the cell surfaces (Bai M, Harfe B, Freimuth P, Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells, J. Virol. 67: 5198-5205, 1993; Wickham T J et al., Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ promote adenovirus internalization but not virus attachment, Cell 73: 309-319, 1993). Viruses that reach the endosomes alter capsid protein structures under acidic conditions, disrupt the endosomes, and infiltrate the cytoplasm. Accordingly, the first step of infection is

the binding of viral fibers to the CAR receptors on the cell surfaces, and the infection range for a vector is considered to be capable of being varied via modification of fibers (Paillard, F., Dressing up adenoviruses to modify their tropism, Hum. Gene Ther. 10: 2575-2576, 1999).

Adenovirus type 35 was first discovered in urine of patients such as those who had undergone kidney transplantation, those who had undergone marrow graft, and those with AIDS. Infection therewith is said to cause acute hemorrhagic cystitis and to infect the kidney. At present, a receptor for adenovirus type 35 infection is not yet known.

Disclosure of the Invention

Examples of known vectors that are used when transfecting a foreign gene into a target cell are adenovirus types 2, 5, and 7 that are infected with humans as a host and chimpanzees-adenoviruse, mouse-adenoviruse, dog-adenoviruse, sheep-adenoviruse and bird-adenoviruse that are infected with non-humans as a host.

However, vectors utilizing adenoviruses as mentioned above have insufficient infectivity depending on the target cell type or insufficient gene transfection efficiency. Thus, such vectors have not been able to accomplish the end desired.

Accordingly, an object of the present invention is to provide an adenovirus vector that has excellent gene transfection efficiency on specific cell lines, particularly on hematopoietic cells, ES cells, pluripotent stem cells, blood stem cells, and tissue stem cells.

The present invention that has attained the above object includes the following.

1. An adenovirus type 35 vector, which is derived from the adenovirus type 35 genome at least by partial or total deletion of the E1 region therefrom.

2. The adenovirus type 35 vector according to 1. above, wherein the E1 protein encoded by the aforementioned E1 region is rendered incapable of being expressed or is functionally defective.

3. The adenovirus type 35 vector according to 1. or 2. above, wherein part of the E1 region is equivalent to the region between nucleotides 367 and 2,917 of the

adenovirus type 35 genome.

4. The adenovirus type 35 vector according to 1. or 2. above, wherein part of the E1 region is equivalent to the region between nucleotides 367 and 3,375 of the adenovirus type 35 genome.

5. The adenovirus type 35 vector according to 1. above, wherein the E3 region is further partially or totally deleted from the adenovirus type 35 genome.

6. The adenovirus type 35 vector according to 5. above, wherein part of the E3 region is equivalent to the region between nucleotides 2,776 and 29,732 of the adenovirus type 35 genome.

7. The adenovirus type 35 vector according to any one of 1. to 6. above, wherein a foreign gene is inserted into a site that lacks part or all of the E1 and/or E3 regions.

8. A method for producing an adenovirus type 35 vector comprising the following steps of:

(1) preparing an adenovirus type 35 vector derived from the adenovirus type 35 genome by partially or totally deleting the E1 region therefrom;

(2) allowing the prepared vector to infect and proliferate in adenovirus E1 protein- and E4 protein-expressing cells; and

(3) recovering the proliferated vectors.

9. The method for producing an adenovirus type 35 vector according to 8. above, wherein step (1) further comprises a step of partially or totally deleting the E 3 region.

10. The method for producing an adenovirus type 35 vector according to 8. above, which further comprises a step of inserting a foreign gene into a deleted site between step (1) and step (2).

11. The method for producing an adenovirus type 35 vector according to 8. above, wherein the cell employed in step (2) is of the 293-cell line.

12. An adenovirus type 35 vector, which is obtained by the method for producing an adenovirus type 35 vector according to any one of 8. to 11. above.

13. A method for producing an adenovirus type 35 vector comprising the following steps of:

(1) preparing part of the adenovirus type 35 genome that lacks part or all of the El region;

(2) ligating the part of the adenovirus type 35 genome to the remaining portion of the adenovirus type 35 genome and thereby preparing an adenovirus type 35 vector derived from the adenovirus type 35 genome by partial or total deletion of the El region therefrom;

(3) allowing the prepared vector to infect and proliferate in adenovirus El protein- and E4 protein-expressing cells; and

(4) recovering the proliferated vectors.

14. The method for producing an adenovirus type 35 vector according to 13. above, wherein step (1) or (2) further comprises a step of partially or totally deleting the E 3 region.

15. The method for producing an adenovirus type 35 vector according to 13. above, wherein step (1) further comprises a step of inserting a foreign gene into a deleted site.

16. The method for producing an adenovirus type 35 vector according to 13. above, wherein the cell employed in step (3) is of the 293-cell line.

17. The method for producing an adenovirus type 35 vector according to 13. above, wherein the part of the adenovirus type 35 genome mentioned in (1) is equivalent to a region lacking the region between nucleotides 367 to 2,917 or that between nucleotides 367 to 3,375 of the region between nucleotides 1 to 7,932.

18. An adenovirus type 35 vector, which is obtained by the method for producing an adenovirus type 35 vector according to any one of 13. to 17. above.

19. A method for gene transfection, wherein the adenovirus type 35 vector according to any one of 1. to 7., 12., and 18. above is allowed to infect a target cell.

20. The method for gene transfection according to 19. above, wherein the target cell is selected from the group consisting of hematopoietic cells, blood stem cells, ES cells, pluripotent stem cells, and tissue stem cells.

21. The method for gene transfection according to 19. above, wherein the target

cell is a $CD34^+$ cell.

Hereafter, the present invention is described in detail.

The adenovirus vector according to the present invention is derived from the adenovirus type 35 genome at least by partial or total deletion of the E1 region therefrom. In the following description, a site or region lacking part or all of the E1 region may be referred to as the "E1-deleted region." In the present invention, the term "lacking part or all of the E1 region" refers to, but is not limited to, a situation where the E1 protein encoded by the E1 region is rendered incapable of being expressed or is functionally defective. Also, the adenovirus vector according to the present invention may consist of part of the adenovirus type 35 genome as long as the E1-deleted region is present. Alternatively, the adenovirus vector according to the present invention may consist of the entire adenovirus type 35 genome having the E1-deleted region. In the description and drawings below, adenovirus type 35 may be abbreviated as "Ad35."

An adenovirus vector having the E1-deleted region and consisting of part of the adenovirus type 35 genome can be obtained by, for example, cleaving a fragment comprising the E1 region of the adenovirus type 35 genome with a restriction enzyme, deleting the E1 region from the cleavage fragment to obtain part of the adenovirus type 35 genome, ligating the resultant to a given vector, and transfecting the ligation product into E1 protein- and E4 protein- expressing 293-cell lines, followed by recovery from the cells.

A specific example of the adenovirus vector consisting of part of the adenovirus type 35 genome is one consisting of a nucleotide sequence lacking the region between nucleotides 367 to 2,917 (E1-deleted region) of the region between nucleotides 1 to 7,932 of the adenovirus type 35 genome. Numbers in the nucleotide sequence of the adenovirus type 35 genome are based on the nucleotide sequence that is registered in the Gene Bank database under the accession number AX049983. The nucleotide sequence of the adenovirus type 35 genome is shown in SEQ ID NO: 1.

The E1 region of the adenovirus type 35 genome refers to a region that encodes

the E1 protein, which is essential for proliferation of common adenoviruses. Part of the E1 region of the adenovirus type 35 genome is equivalent to the region between nucleotides 367 and 2,917 in the nucleotide sequence of the adenovirus type 35 genome and is present in a 2,550 bp fragment that is generated upon treatment of the adenovirus type 35 genome with restriction enzymes *Acc*I and *Pac*I. Alternatively, part of the E1 region is equivalent to the region between nucleotides 367 and 3,375 in the nucleotide sequence of the adenovirus type 35 genome and is present in a 3,008 bp fragment that is generated upon treatment of the adenovirus type 35 genome with restriction enzymes *Acc*I and *Bam*HI. Further, the E1 region is constituted by the E1a and E1b regions.

In particular, the term "E1-deleted region" refers to an E1 protein-encoding region that is functionally defective. The term "functionally defective" refers to the fact that, for example, the E1 protein is not allowed to express in a manner such that it functions in a host cell. Accordingly, the adenovirus vector according to the present invention does not necessarily lack the entire E1 region. Instead, it may have part of the E1 region. Specifically, the adenovirus vector according to the present invention may comprise part of the E1 region of the adenovirus type 35 genome as long as the E1 protein that functions in a host cell is not expressed. The "E1-deleted region" may lack all or part of the E1a or E1b region, may lack all of both regions, or may lack a portion that spans both regions, as long as an E1 protein-encoding region is functionally defective.

The adenovirus vector according to the present invention may be constituted by part or all of the adenovirus type 35 genome that lacks the E3 region as well as the E1 region. The E3 region in the adenovirus type 35 genome can be deleted by treatment the adenovirus type 35 genome with *Eco*RI and *Bam*HI, and removing a site equivalent to the region between nucleotides 27,760 and 29,732. Use of the adenovirus type 35 genome that lacks the E3 region as well as the E1 region enables insertion of a large-size foreign nucleotide sequence into the E1-deleted region.

Further, the adenovirus vector according to the present invention has the E1-deleted region and may have attenuated immune responses due to partial deletion of

genes that are present in the adenovirus type 35 genome. In other words, the adenovirus vector according to the present invention may be a so-called "gutted" ("gutless") adenovirus vector having the E1-deleted region and consisting of part of the adenovirus type 35 genome.

The recombinant adenovirus vector according to the present invention has a foreign nucleotide sequence in the E1-deleted region and comprises the entire adenovirus type 35 genomes excluding the E1-deleted region. This recombinant adenovirus vector can be prepared using the adenovirus vector according to the present invention. Specifically, such recombinant vector can be prepared from an adenovirus vector having the E1-deleted region and having part of the adenovirus type 35 genome or an adenovirus vector having the E1-deleted region and having the entire adenovirus type 35 genome.

When preparing the recombinant adenovirus vector according to the present invention with the utilization of an adenovirus vector having the E1-deleted region and having part of the adenovirus type 35 genome, a foreign nucleotide sequence is first inserted into the E1-deleted region of the adenovirus vector, the resultant is ligated to the remaining portion of the adenovirus type 35 genome, and the ligation product is transfected into E1 protein- and E4 protein-expressing 293-cell, followed by recovery from the cells. Thus, the aforementioned recombinant adenovirus vector having a foreign nucleotide sequence can be prepared. In the case of an adenovirus vector consisting of the entire adenovirus type 35 genome having the E1-deleted region, a foreign nucleotide sequence can be inserted into the E1-deleted region to prepare the recombinant adenovirus vector having the aforementioned nucleotide sequence.

In general, when the E1 region is deleted from adenovirus, it cannot be proliferated in cells other than the E1 protein-expressing cell (for example, those of the 293-cell). For example, the E1 region-deleted adenovirus type 5 that is used as a vector for gene transfection can proliferate in cells of the 293-cell line, although it cannot proliferate in the target cell of gene transfection.

When the E1 region is deleted from adenovirus type 35, the adenovirus can

proliferate in adenovirus type 5 E1 and E4 protein-expressing cells. However, the adenovirus cannot proliferate in cells in which the E1 protein is expressed but the E4 protein is not expressed. Specifically, proliferation characteristics of the E1 region-deleted adenovirus type 5 are different from those of the E1 region-deleted adenovirus type 35.

A foreign nucleotide sequence to be inserted into the E1-deleted region is not particularly limited, and any nucleotide sequence may be employed. Examples thereof include a nucleotide sequence that encodes a protein or peptide, a nucleotide sequence that is present in the regulatory region of a given gene, and a nucleotide sequence to which a given protein can bind. Particularly preferably, a gene that is effective or supposed to be effective for what is termed "gene therapy" is used as a foreign nucleotide sequence. More preferably, gene therapy includes treatment or prevention of diseases relating to hematopoietic cells, ES cells, pluripotent stem cells, blood stem cells, or tissue stem cells and gene therapy aimed at ameliorating symptoms caused by hematopoietic cells, ES cells, pluripotent stem cells, blood stem cells, or tissue stem cells.

When a nucleotide sequence having a promoter sequence that regulates gene expression, a gene that encodes luciferase, and a poly A sequence, in that order, is employed as a foreign nucleotide sequence, gene transfection efficiency to the target cell can be evaluated by assaying luciferase activity. When a gene that encodes a green fluorescent protein (so-called GFP) is used instead of the gene that encodes luciferase, gene transfection efficiency to the target cell can be evaluated by assaying the green fluorescence level in the target cell.

Preferably, hematopoietic cells such as $CD34^+$ cells or hematopoietic stem cells are used as the target cells of gene transfection. A receptor of the hematopoietic cell for adenovirus type 35 infection is unknown. A recombinant adenovirus vector having a foreign nucleotide sequence of luciferase, GFP, or another gene can assay the amount of foreign nucleotide sequence introduced. Thus, it is useful when searching for a receptor for infection in hematopoietic cells. Examples of the target cell for gene

transfection that can be employed include ES cells, pluripotent stem cells, blood stem cells, and tissue stem cells.

It is known that adenovirus type 35 is highly compatible with human $CD34^+$ cells and that a chimera vector (Ad5/F35) comprising part of a fiber region of adenovirus type 35 in the capsid of adenovirus type 5 efficiently transfects genes to human $CD34^+$ cells (Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G. and Lieber, A., 2000, Efficient gene transfer into human $CD34^{(+)}$ cells by a retargeted adenovirus vector, J. Virol. 74, 2567-2583).

The recombinant adenovirus vector according to the present invention can also infect hematopoietic cells such as human $CD34^+$ cells with high compatibility. Furthermore, the recombinant adenovirus vector according to the present invention can efficiently introduce a nucleotide sequence that encodes a foreign peptide to hematopoietic cells.

The recombinant adenovirus vector according to the present invention is useful for repeated administration. When a common adenovirus type 5 vector is used for repeated administration to a subject animal, it is known that gene transfection efficiency is lowered as the number of administrations is increased as a result of an antigen-antibody reaction within the subject animal.

Accordingly, genes can be transfected into the subject animal with excellent efficiency at the second or later administration by, for example, conducting the first administration to the subject animal using a common adenovirus type 5 vector and then conducting the second or subsequent administrations to the subject animals using the recombinant adenovirus vector according to the present invention.

This description includes part or all of the contents as disclosed in the description and/or drawings of Japanese Patent Application No. 2002-164015, which is a priority document of the present application.

Brief Description of the Drawings

Fig. 1 shows a process of constructing a plasmid pAdMS1 having the adenovirus type 35 genome.

Fig. 2 shows a process of constructing Ad35GFP having a GFP expression cassette.

Fig. 3 is a cause-and-effect diagram showing fluorescence intensities assayed as a result of gene transfection using Ad5GFP, Ad5F35GFP, and Ad35GFP.

Fig. 4 is a cause-and-effect diagram showing luciferase activities assayed as a result of gene transfection using Ad5L, Ad5F35L, and Ad35L.

Fig. 5 is a cause-and-effect diagram showing the results of the repeated *in vivo* administration experiment using Ad35.

Preferred Embodiments of the Invention

The present invention is hereafter described in more detail with reference to the following examples, although the technical scope of the present invention is not limited thereto.

[Example 1]

Preparation of adenovirus type 35 vector

· Preparation of plasmid

A plasmid (pAdMS1) having the *Sbf*I recognition sequence and the *Not*I recognition sequence at the both terminuses of the adenovirus type 35 genome was first prepared. Fig. 1 shows the preparation procedure thereof.

When preparing pAdMS1, adenovirus type 35 (ATCC No. VR-718) was first obtained from the American Type Culture Collection (ATCC). The thus obtained adenovirus type 35 was proliferated in HeLa cells and purified via CsCl density gradient centrifugation. Adenovirus type 35 was then processed with proteinase K, thereby isolating adenovirus type 35 genomic DNA. An *Sbf*I site was next added to the 5' terminus of the isolated genomic DNA. The genomic DNA was processed with *Pac*I and the resulting 2,917 b fragment was cloned into the *Sbf*I/*Pac*I site of a pFS2 vector.

The pFS2 vector having the 2,917 b fragment cloned therein was designated as pFS2-Ad35-5. The cloned 2,917 b fragment contained the 5' inverted terminal repetition (ITR) of adenovirus type 35. Further, a *Not*I site was added to the 3' terminus of the isolated genomic DNA. The genomic DNA was then processed with *Eco*RI. The resulting 7,034 b fragment was cloned into the *Eco*RI/*Not*I site of a pHM15 vector. The pHM15 vector having the 7,034 b fragment cloned therein was designated as pHM15-Ad35-1. The cloned fragment (approximately 7 kb) contained the 3' ITR of the adenovirus type 35 genome.

The pFS2 vector used herein was prepared by replacing the *Xba*I/*Xho*I/*Eco*RI/*Kpn*I/*Sma*I/*Csp*45I/*Cla*I/*Hind*III/*Bam*HI/*Sac*I site of pGEM7Zf (+) (Promega) with the *Sbf*I/*Swa*I/*Pac*I/*Asc*I/*Sgf*I/*Not*I site. The pHM15 vector was prepared by replacing the I-*Ceu*I/*Hind*III/*Sph*I site and the PI-*Sce*I site of pHM5 (Mizuguchi, H. and Kay, M. A.: A simple method for constructing E1 and E1/E4 deleted recombinant adenovirus vector: Hum. Gene Ther., 10, 2013-2017, 1999) with the *Xba*I/*Avr*II/*Nhe*I/*Spe*I/*Not*I site and the *Pvu*II/*Apa*I/*Spe*I/*Nhe*I/*Avr*II/*Xba*I site, respectively.

The thus obtained pFS2-Ad35-5 and pHM15-Ad35-1 were then digested with *Bam*HI and *Not*I, respectively. Accordingly, pFS2-Ad35-5 was linearized by being cleaved at the *Bam*HI recognition site in a region derived from genomic DNA. Separately, a *Bam*HI-*Not*I fragment containing a region derived from the genomic DNA was cleaved out of pHM15-Ad35-1 in accordance with a conventional technique. The vector obtained by ligating the linearized pFS2-Ad35-5 fragment to the cleaved *Bam*HI-*Not*I fragment was designated as pFS2-Ad35-6.

The obtained pFS2-Ad35-6 was linearized by digestion with *Bam*HI. The linearized pFS2-Ad35-6 and adenovirus type 35 genomic DNA were transformed into an *E. coli* BJ5183 strain. Thus, homologous recombination took place between pFS2-Ad35-6 and adenovirus type 35 genomic DNA in the *E. coli* BJ5183 strain. Subsequently, a plasmid pAdMS1 was prepared by extracting a plasmid from the *E. coli* BJ5183 strain in accordance with a conventional technique.

A GFP-expressing recombinant adenovirus vector was prepared using a plasmid pAdMS1. Fig. 2 shows the preparation procedure thereof. First, adenovirus type 35 genomic DNA was digested with *Pac*I and *Asc*I, thereby cleaving out a *Pac*I/*Asc*I fragment equivalent to the region between nucleotides 2,917 to 7,932 of the aforementioned genomic DNA. Secondly, pFS2-Ad35-5 was digested with *Acc*I and *Pac*I, thereby removing an *Acc*I/*Pac*I fragment equivalent to the region between nucleotides 367 to 2,917 of adenovirus type 35 genomic DNA and converting an *Acc*I recognition site into a *Pac*I recognition site. The resultant was further digested with *Pac*I and *Asc*I and ligated to a *Pac*I/*Asc*I fragment equivalent to the region between nucleotides 2,917 to 7,932 of adenovirus type 35 genomic DNA. This enabled construction of a plasmid consisting of a nucleotide sequence derived from the nucleotide sequence consisting of nucleotides 1 to 7,932 of the genomic DNA by deletion of nucleotides 367 to 2,917 and conversion of the *Acc*I recognition site into the *Pac*I recognition site. The thus obtained plasmid was designated as pFS2-Ad35-7.

Next, a GFP expression cassette in which the cytomegarovirus (CMV) promoter, the GFP gene, and the bovine growth hormone (BGH) poly A sequence were ligated in that order was integrated and then cloned into the *Pac*I recognition site of pFS2-Ad35-7. The resulting plasmid was designated as pFS2-Ad35-7-GFP1. The pFS2-Ad35-7-GFP1 and pAdMS1 (Fig. 1) were digested with *Sbf*I and *Asc*I, respectively. A fragment containing a GFP expression cassette of pFS2-Ad35-7-GFP1 was ligated to a fragment prepared by removing an *Sbf*I/*Asc*I fragment (equivalent to the region between nucleotides 1 to 7,932 of the genomic DNA) from a plasmid pAdMS1. As a result, a plasmid pAdMS1-GFP1 that contains adenovirus type 35 genomic DNA having a GFP expression cassette incorporated in the E1-deleted region was constructed.

Also, a plasmid pAdMS1-L2 that contains adenovirus type 35 genomic DNA having a luciferase expression cassette incorporated in the E1-deleted region was constructed in a manner similar to that used for pAdMS1-GFP1.

· Construction of recombinant adenovirus vector

The thus obtained pAdMS1-GFP1 and pAdMS1-L2 were constructed as recombinant adenovirus vectors in the following manner. That is, pAdMS1-GFP1 and pAdMS1-L2 were first digested with *Sbf*I and *Not*I, respectively, and then transfected into the 293-cell line (VK10-9), which simultaneously expresses the E1 and E4 genes. Cytopathic effects were observed 10 to 14 days after the transfection, and amplification of the viruses derived from the plasmids was confirmed. The viruses derived from the plasmids were purified in accordance with a conventional technique (Lieber, A. et al., J. Virol. 70, 8944-8960, 1996).

As a result, the yield of the virus derived from pAdMS1-GFP1 (Ad35GFP) was approximately 1.5 ml at cell densities of 10^{11} virus particles/ml. This yield was substantially equivalent to or somewhat smaller than that of adenovirus type 5. The virus derived from pAdMS1-L2 (Ad35L) was purified in the same manner as described above. Thus, the adenovirus type 35 vector having a GFP expression cassette incorporated therein and the adenovirus type 35 vector having a luciferase expression cassette incorporated therein were constructed.

As the control, vectors having a GFP expression cassette or a luciferase expression cassette incorporated therein, Ad5GFP and Ad5F35GFP, were constructed using a normal adenovirus type 5 vector (Ad5) and the adenovirus type 5 vector (Ad5F35), the fiber region of which had been substituted with that of adenovirus type 35.

The ratio of the plaque forming unit (PFU) to the virus particle titer was 1:133 for Ad35GFP, 1:24 for Ad5F35GFP, 1:56 for Ad5GFP, 1:225 for Ad35L, 1:13 for Ad5F35L, and 1:13 for Ad5L. The PFU was measured in accordance with the method disclosed in Kanegae Y. et al., Jpn. J. Med. Sci. Biol., 1994, 47: 157-166. The virus particle titer was measured in accordance with the method disclosed in Maizel Jv. et. al., Virology. 1968, 36: 115-125.

· Experimentation of gene transfection into hematocytes

Gene transfection into hematocytes was carried out using the recombinant

adenovirus vectors (Ad35GFP and Ad35L) constructed above. Human $CD34^+$ cells (BioWhittaker) were employed as hematocytes. According to the manufacturer's instructions, 95% or more of the cells were CD34 positive.

16 to 20 hours before the initiation of the gene transfection experiment, human $CD34^+$ cells were converted from a cryopreserved state, and then were dissolved in StemSpan™ 2000 (StemCell Technologies, Inc). The StemSpan™ 2000 was used for the experiment in the form of a mixture with cytokine cocktail StemSpan™ CC100 (human flt-3 ligand (100 ng/ml), human stem cell factor (100 ng/ml), human interleukin-3 (20 ng/ml), and human interleukin-6 (20 ng/ml)). Thereafter, human $CD34^+$ cells were inoculated on a 24-well plate at a cell density of 1×10^5 cells/well. Ad35GFP, Ad5GFP, and Ad5F35GFP were diluted to cell densities of 3 PFU/cell, 30 PFU/cell, and 300 PFU/cell, respectively. Gene transfection into human $CD34^+$ cells was carried out employing Ad35GFP, Ad5GFP, and Ad5F35GFP at the aforementioned densities, respectively.

48 hours thereafter, expression of the GFP gene in human $CD34^+$ cells was analyzed by flow cytometry using a FACScalibur flow cytometer equipped with the CellQuest software (Becton Dickinson). The results are shown in Fig. 3.

When Ad35L, Ad5L, and Ad5F35L were used, human $CD34^+$ cells were inoculated on a 96-well plate at a cell density of 1×10^4 cells/well. Ad35L, Ad5L, and Ad5F35L were diluted to cell densities of 3, 30, 100, and 300 PFU/cell and to cell densities of 300, 3,000, 6,000, and 9,000 vector particles/cell, respectively. Gene transfection into human $CD34^+$ cells was carried out employing Ad35L, Ad5L, and Ad5F35L at the aforementioned densities, respectively. 48 hours thereafter, luciferase gene expression in human $CD34^+$ cells was evaluated using the luciferase assay system (PicaGene LT 2.0, Toyo Ink). The results are shown in Fig. 4. In Fig. 4, "B-1" shows the results attained when the viruses were used at cell densities of 3, 30, 100, and 300 PFU/cell, respectively, and "B-2" shows the results attained when the viruses were used at cell densities of 300, 3,000, 6,000, and 9,000 vector particles/cell, respectively.

· Evaluation of gene transfection into hematocytes using each virus

The results shown in Fig. 3 show that the use of Ad35GFP can result in GFP gene transfection with much higher efficiency than the use of Ad5GFP and Ad5F35GFP. Particularly when Ad35GFP was used at a cell density of 300 PFU/cell, the GFP gene was expressed in 59% of human $CD34^+$ cells. At the same density, the GFP gene was expressed in 5% and 52% of human $CD34^+$ cells with the use of Ad5GFP and Ad5F35GFP, respectively. The mean fluorescence intensity (MFI) with the use of Ad35GFP was 10 to 70 times larger than that with the use of Ad5GFP, and 2 to 3 times larger than that with the use of Ad5F35GFP.

The results shown in "B-1" of Fig. 4 indicate that the level of luciferase expression with the use of Ad35L was 1,000 to 3,000 times larger than that with the use of Ad5L and 15 to 100 times larger than that with the use of Ad5F35L. Unlike the case of adenovirus type 5, the 293-cell line expressing the E4 gene product may not completely produce adenovirus type 35. Thus, there is a possibility that gene transfection efficiency measured with the PFU titer may be underestimated for Ad35L as shown in "B-1" of Fig. 4. Therefore, gene transfection efficiency was evaluated at cell densities of 300, 3,000, 6,000, and 9,000 vector particles/cell. As a result, gene transfection efficiency of Ad35L was found to be higher than that of Ad5L and that of Ad5F35L at a cell density of 3,000 vector particles/cell or higher, as shown in "B-2" of Fig. 4.

Accordingly, gene transfection efficiency into human $CD34^+$ cells was much better when Ad35 was used than when Ad5 or Ad5F35 was used. Thus, gene transfection using Ad35 was found to be particularly effective on hematocytes such as hematopoietic stem cells.

· Repeated *in vivo* administration experiment using Ad35

In this embodiment, the usefulness of Ad35 was examined by a repeated *in vivo* administration experiment consisting of a first administration with the use of Ad5 and a subsequent second administration with the use of Ad35 for gene transfection.

Mice (C57B16, Japan SLC) were used as experimental animals. In addition, Ad5L was employed for the first administration. Ad35 (Ad35RSVSEAP1) having a human secretory enzymatic alkaline phosphatase (SEAP) expression cassette (RSVSEAP1) incorporated therein was used for the second administration in accordance with the method described above. In RSVSEAP1, the Rous sarcoma virus (RSV) promoter, the SEAP gene, and the BGH poly A sequence were ligated in that order. As the control, Ad5 (Ad5RSVSEAP1) having RSVSEAP1 incorporated therein was used for the second administration. The repeated *in vivo* administration experiment was conducted according to the following procedure.

Ad5L was first administered to the caudal veins of mice in amounts of 1.5×10^{10} vector particles/mouse. 14 days after the first administration, Ad5RSVSEAP1 or Ad35RSVSEAP1 was administered intramuscularly to the mice in amounts of 1.5×10^{10} vector particles/mouse. Blood was sampled from the ophthalmic vessels of mice 2 days after the second administration to measure the amount of serum SEAP. The amount of SEAP was measured using the Great EscAPe™ SEAP Chemiluminescence Detection Kit (Clontech). Ad5RSVSEAP1 or Ad35RSVSEAP1 was administered intramuscularly to the control group via the second administration while no first administration took place.

The results are shown in Fig. 5. The data in Fig. 5 are shown in terms of the mean value of four experimental measures ± S.D. The vertical axis represents the amount of SEAP, which is a relative value attained when the SEAP amount of the control group is determined to be 100%. When Ad5 was used for the first and second administrations, the amount of SEAP resulting from the second administration fell to 5% or less that of the group to which no first administration had been made (the control group). In contrast, when Ad5 was used in the first administration and Ad35 was used in the second administration, the amount of SEAP resulting from the second administration was almost equal to that of the group to which no first administration had been made (the control group).

As described above, employment of commonly used adenovirus vectors, such as Ad5 for the first administration and Ad35 for the second or subsequent administrations,

can prevent a disadvantage whereby the gene transfection efficiency is lowered as the number of administration is increased. Thus, it has been proved that gene transfection can be carried out with excellent efficiency at the second or subsequent administration.

All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

Industrial Applicability

As is apparent from the foregoing detailed description, the present invention can provide an adenovirus vector that has excellent gene transfection efficiency on specific cell lines, particularly on hematopoietic cells, a method for producing such vector, and a method for gene transfection using such vector.